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(54) Title: NEW CELL ADHESION PEPTIDES

(57) Abstract

A cell adhesion peptide is disclosed, which may be a polypeptide, including an amino acid sequence comprising X-Asp-Y-(A)n-Phe, where X and Y are chosen from Ala, Leu, Ile and Val, A is any amino acid and n is in the range 3 to 10, in which at least a sub-sequence of the said sequence is adherent for MOLT-4 human lymphoblastic leukaemia, A375-SM human metastatic melanoma or H1080 human fibrosarcoma cells.

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New cell adhesion peptides.

This invention relates to new peptides, which have the properties of regulating or controlling cell adhesion.

It is known that cell adhesion occurs ubiquitously within the body and is central to many normal events, such as wound healing and cell migration during development. However, it also causes or contributes to many undesirable conditions or effects, for example the pathogenesis of many of the major diseases. These include thrombosis, inflammation, auto-immune diseases, malignant cancer and arthritis.

It is clearly vital to life that the function of cell adhesion should not be prevented in all circumstances, as this would destroy the life processes. It is, however, very desirable to have some way in which the desirable and undesirable forms of cell adhesion can be distinguished from each other and to have means which allow this distinction to be used so that treatment agents can be administered to interfere with or impede the undesirable ones without unduly affecting the desirable ones.

It is known that cells can interact with short amino acid sequences in the active sites of adhesion proteins such as fibronectin. One such sequence has been identified, the tetramer sequence R-G-D-S (in which R = arginine; G = glycine; D = aspartic acid; S = serine), (US 4,578,079). These sequences are contained within several proteins and represent a common adhesive signal.

According to the present invention there is provided a cell adhesive peptide, which may be a polypeptide, useful as a selective antagonist to the agents and processes of cell adhesion, including a novel adhesive signal, namely an amino acid sequence comprising X-Asp-Y-(A)n-Phe, where X and Y are chosen from Ala, Leu, Ile, and Val, A is any amino acid and n is in the range 3 to 10, in which at least a sub-sequence of the said sequence is adherant for MOLT-4 human lymphoblastic leukaemia, or A375-SM human metastatic melanoma or H1080 human fibrosarcoma cells.

The preferred sequence is that designated as "L-D-V" (leucine-aspartic acid-valine) and peptides containing this are able to promote MOLT-4, A375-SM or H1080 cell adhesion while inhibiting adhesion to the parent molecules, ie to the natural adhesion protein containing the adhesive sequence. By blocking cell receptors, the peptide inhibits natural adehsion proteins from binding to the cell.

Alternative useful sequences are those designated as L-D-L (leucine-aspartic acid-leucine) or I-D-A (isoleucine-aspartic acid-alanine).

The sequence of amino acid units in a peptide is normally written as starting from the unit with the "free N" (as the $-NH_2$ group) and running to that with the "free C" (as the -COOH group). This convention is followed and used throughout this specification for designating the sequences of amino acid units in the peptide compounds of the invention.

Also, the letters used above (e.g. "D" for aspartic acid) are those already well established and conventionally used in the art for the designation of the various amino acids which are found in naturally occurring peptides.

We define the peptide sequence containing a novel adhesive signal of the present invention in terms of the number of amino acid units and part of the sequence of such units within them. The number of amino acid units may vary according to particular requirements, for example of manufacture and activity, but we prefer that the number of amino acid units should be in the range 15 to 40, and still more preferably in the range 20 to 30. The number may be larger or smaller than these ranges, but these tend to be less useful

and/or more difficult to make or use; building up a large peptide molecule is more expensive as the number of amino acid units increases, so the economic factor then becomes more important.

A convenient number of amino acid units is 19 (the compounds then being conveniently referred as as "19-mers"), but this number is not to be taken as the only number permissible.

Examples of some specific compounds or sequences according to the present invention include :-

1. H.G.P.E.I.L.D.V.P.S.T.V.Q.K.T.P.F.V.T.
2. V.V.I.D.A.S.T.A.I.D.A.P.S.N.I.R.F.L.A.
3. A.P.D.K.T.L.I.L.D.V.P.P.G.V.E.K.F.Q.L.
4. R.S.L.T.L.D.V.Q.G.R.E.N.N.K.D.Y.F.S.P.
5. T.A.S.V.L.V.T.V.L.D.V.N.E.P.P.V.F.V.P.
6. F.S.C.R.T.E.L.D.L.R.P.Q.G.L.E.L.F.E.N.
7. F.S.C.L.A.V.L.D.L.M.S.R.G.G.N.I.F.H.K.
8. C.E.K.M.E.N.A.E.L.D.V.P.I.Q.S.V.F.T.R.
9. C.S.Q.P.L.D.V.I.L.L.L.D.G.S.S.S.F.P.A.
10. D.Q.D.A.T.M.S.I.L.D.I.S.M.M.T.G.F.A.P.
11. V.G.L.S.G.M.A.I.A.D.V.T.L.L.S.G.F.H.A.
12. R.S.A.S.N.M.A.I.V.D.V.K.M.V.S.G.F.I.P.
13. P.I.I.D.V.A.P.L.D.V.G.A.P.D.Q.E.F.G.F.
14. P.I.L.D.I.A.P.L.D.I.G.G.A.D.Q.E.F.G.L.

15. P.I.I.D.I.A.P.M.D.I.G.G.P.E.Q.E.F.G.V.

16 P.I.V.D.I.A.P.Y.D.I.G.G.P.D.Q.E.F.G.V.

17. G.V.T.D.A.A.K.A.C.N.L.D.V.I.L.G.F.D.G.

18. S.P.P.G.Y.T.I.L.D.V.D.A.N.A.M.L.F.V.G.

19. S.P.G.P.S.K.V.L.D.I.N.N.S.T.L.M.F.V.G.

20. G.V.E.N.V.T.I.Q.L.D.L.E.A.E.F.J.F.T.H.

21. T.W.K.P.Y.D.A.A.D.L.D.P.T.E.N.P.F.D.L.

In these, the amino acid sequences of importance, specified above, are indicated by underlining for ease of recognition.

The specified amino acid unit sequence may thus be seen as present as part of the structure of a larger polypeptide (i.e. "buried" within a polypeptide structure) having more amino acid units, but accessible to a cell receptor and thus able to bind thereto. Other proteins and peptide sequences may occur in nature which contain the amino acid sequence of the present invention, however, this does not necessarily mean they are adhesion proteins/peptides and may not therefore be adherant for MOLT-4, A375-SM or H1080 cells.

The peptide sequences of the present invention may be made by conventional peptide-forming reactions and processes, well known in the art and used to build up the desired sequence from the individual component amino acids or appropriate combinations of them. For

example, they may be made using automated synthesisers which are commercially available, or the peptide sequence may be synthesised using recombinant DNA technology.

There are several different machines that are used for peptide synthesis, and they all perform the same function in essentially the same manner. They use conventional chemical reactions to build up the polypeptide structure from successive amino acids. In these, peptides are usually built up by adding derivatised and blocked amino acids sequentially to the first (C-terminal) amino acid which has been previously immobilised on an inert bead "carrier". The synthesis occurs sequentially from the C-terminus by successive addition of blocked amino acids and finally the finished peptide is simultaneously cleaved off from the bead and de-blocked, prior to purification. This procedure of solid phase synthesis on resin bead supports uses either t-boc or f-moc chemistries. Peptides will be cleaved from the resin by treatment with anhydrous hydrofluoric acid (t-moc) or by trifluoroacetic acid (f-boc) and purified by reverse-phase HPLC (high pressure liquid chromatography).

According to the present invention, there are also provided new compositions and methods for the modification or control of the adhesive properties of biological cells which comprise treating them with one or more of the new peptides defined above.

In use, the compounds of the present invention may be made into pharmaceutical compositions by combining one or more of the compounds defined above with one or more pharmaceutically-acceptable excipients and/or diluents.

Usually this will be by formulation in any manner or composition appropriate and conventional for the administration of peptide drugs. Thus the compounds of the present invention may be administered intravenously or subcutaneously, or they may be incorporated into dressing, wash solutions and the like. Commonly, they may be solubilised or dispersed in normal saline.

The peptide sequences of the present invention are useful as selective antagonists to the agents and processes of cell adhesion but may be effective in different ways and in different circumstances, depending for example upon such factors as the particular natural processes involved.

Thus, they can be active in various leukocyte functions, for example by flooding the system with peptides to block adhesion protein receptors on cells, they aid in blocking leukocyte extravasation during inflammation, lymphocyte cell-cell recognition during responses to antigens and in inhibiting platelet aggregation and fibrosis. Hence they can be used to alleviate conditions in which these mechanisms are involved, and particularly in inflammatory or coagulatory conditions. Thus they may be used for treatment of, for example, inflammatory lesions in vivo, rheumatoid arthritis, asthma, inflammatory bowel disease, sepsis, prevention of graft rejection, reperfusion of cardiac tissue after myocardial infarction and dermatoses, and the like.

The peptide sequences of the present invention are primarily useful for their effects on animal cells, but their application is not necessarily restricted to these and can be relevant for other types of biological cells according to the particular amino acid sequences required for optimum effect. The mode of action is not fully understood, but is believed to be either through mediation of the adhesion and migration of various leukocyte subsets from the bloodstream into the inflamed tissue, or possibly by interference with the initial adhesion of leukocytes to the endothelial lining of the blood vessel and impairing the ability of the leukocytes to generate an inflammatory response.

The invention will be further apparent from the following description with reference to several figures of the accompanying drawings, which show, by way of example only, one form of the invention embodying same.

Of the drawings:-

Figure 1 shows attachment of MOLT-4 cells to peptides.

Figure 2 shows the effect of anti-integrin antibodies on peptide mediated MOLT-4 cell attachment and A375-SM cell spreading.

In order to test the ability of peptides containing the novel adhesion signal of the present invention to adhere to cells, two cell adhesion assays were used.

The first assay measures simple attachment of cells to the peptide which is first immobilised on the surface of plastic wells, and the second measures the ability of the cells to attach and then spread out and flatten on to a peptide immobilised in the same way. These are conveniently referred to as "attachment" and "spreading" assays respectively.

For the attachment assay a MOLT-4 human lymphoblastic leukaemia cell line was used, and for the spreading assay an A375-SM human matastatic melanoma cell line was used.

In both assays, the peptide is not used in free solution but is first immobilised onto a plastic substrate. However, the peptide is not coated onto the plastic directly, but prior to the experiment is covalently coupled to normal rabbit IgG and then the resulting conjugate is coated. This is done because large protein molecules bind much better to plastic and because the direct binding of peptides may mask their active sites.

However, to test the adhesion properties of proteins containing the novel adhesion signal, these may be coated directly onto the plastic.

In both the cell attachment and cell spreading assays, peptide conjugates were coated after dilution 1:10 with phosphate-buffered saline. Anti-receptor antibodies were then tested for their abilities to block the attachment and spreading induced by the conjugates.

Preparation of Conjugates

Covalent coupling of peptides to IgG via their N-terminal cysteine residues was accomplished by cross-linking with the hetero-bifunctional agent N-succinimidyl-3(2-pyridylthio) propionate (SPDP). SPDP was dissolved in ethanol to a concentration of 1.5 mg/ml and mixed with 6 mg/ml of rabbit's IgG in Dulbecco's phosphate-buffered saline to give an SPDP:IgG weight ratio of 1:10 (molar ratio of 50:1). After 30 minutes of incubation at room temperature, unreacted SPDP was removed by gel filtration on a PD10 column equilibrated with Dulbecco's phosphate-buffered saline. The activated IgG was then added to peptides at a peptide:IgG weight ratio of 0.3 to 0.45. After mixing overnight on a rotator at room temperature, free peptides were removed by dialysis against Dulbecco's phosphate-buffered saline.

The peptides used for the two tests were :-

1. C.V.V.I.D.A.S.T.A.I.D.A.P.S.N.I.R.F.L.A. (H1)

2. C.R.S.L.T.L.D.V.Q.G.R.E.N.N.K.D.Y.F.S.P. (VCAM1)

3. C.A.P.D.K.T.L.I.L.D.V.P.P.G.V.E.K.F.Q.L. (CD45)

4. C.H.G.P.E.I.L.D.V.P.S.T.V.Q.K.T.P.F.V.T. (CS12)

5. C.D.E.L.P.Q.L.V.T.L.P.H.P.N.L.H.G.P.E.I.L.D.V.P.S.T. (CS1)

6. C.D.E.L.P.Q.L.V.T.L.P.H.P.N.L.H.G.P.P.V.T.S.E.L.I.D. (CS1scr1)

(Note :- the first amino acid unit in the compounds tested is a "C" (cysteine) unit only to provide a means for making the conjugate.)

Assay 1 : Cell attachment

MOLT-4 human lymphoblastic leukaemia cells were activated by addition of 20 nM phorbol-12,13-dibutyrate to their culture medium for 15 minutes at 37°C, and then re-suspended to 10^7/ml in Dulbecco's MEM containing 20 mM HEPES and 1 mM $MnCl_2$. 100 ul aliquots of the cell suspension were then added to adhesive substrates, ie. peptides, coated onto wells of 96-well tissue culture plates and blocked with heat-denatured BSA to block the non-specific adhesion sites on the plastic surface (coating and blocking are as described for the spreading assay below). The cells were incubated in the wells for 15 minutes at 37°C, and loosely adhered cells were removed by gentle agitation and aspiration. Remaining attached cells were fixed by addition of 100 ul of 5% (v/v) glutaraldehyde, washed with water and stained with 10 ul of 0.1% (w/v) crystal violet in 200 mM MES, pH 6, for 20 minutes at room temperature. Cells were then de-stained with three water washes and dye released by addition of 100 ul of 10% (v/v) acetic acid. Colour was quantitated by measuring absorbance at 570 nm in an ELISA reader and the results converted to percent attachment based on an appropriate standard curve.

The results of this assay are shown in figure 1 and Table 1. The adhesion of MOLT-4 cells to peptides containing the novel adhesive signal increases with increasing peptide concentration, ie. is dose dependent. The optimum peptide concentration resulting in 80 to 100% adhesion of the cells was found to be between 1:1000 and 1:10. A peptide sequence (CS1scr1) which did not contain the adhesive signal failed to attach any MOLT-4 cells (figure 1).

TABLE 1

Attachment %	Peptide VCAM1	Peptide CD45	Peptide CS12
Background	5	5	5
1:30000 dilution	4	9	48
1:10000	9	23	56
1:3000	29	42	80
1:1000	73	53	78
1:300	79	76	83
1:100	99	82	85

All the peptides were able to promote attachment of the cells in a dose-dependent manner.

Assay 2 : Cell Spreading

A375-SM cells from a human metastatic melanoma cell line, were cultured in Eagle's minimal essential medium containing 10% fetal calf serum, minimal essential medium vitamins, non-essential amino acids, sodium pyruvate and glutamine. Cell spreading assays were performed in 96-well microtitre plates. Wells were coated for 60 minutes at room temperature with 100 ul aliquots of conjugated peptides diluted with Dulbecco's phosphate-buffered saline, and then sites on the plastic for non-specific cell adhesion were blocked for 30 minutes at room temperature with 100 ul of 10 mg/ml heat-denatured bovine serum albumin (BSA), after which the BSA was removed. Cell cultures were washed with PBS, and cells were detached with 0.25% trypsin, 0.02% EDTA, then washed again. 50 ul aliquots of A375-SM cells suspended in serum-free Dulbecco's minimum essential medium at a concentration of 4×10^5 cells/ml were added to each well. After incubation for 60 minutes at 37°C in a humidified atmsophere of 5% carbon dioxide, attached cells were fixed with 3% glutaraldehyde, and the percentage of cells adopting a normal, well spread morphology was estimated by counting a total of 300 cells/well in a number of randomly selected fields using phase-contrast microscopy. No cell spreading was observed on wells coated only with heat-denatured bovine serum albumin.

The cell receptor involved in this specific adhesion to the peptide sequence of the present invention has been identified as the integrin alpha-4,beta-1. By blocking the receptor with anti-functional anti-integrin subunit monoclonal antibodies and repeating the cell spreading and cell attachment assays, confirmation that the peptide induced cell attachment and spreading was due to adhesion to this receptor may be acheived if the presence of antibody inhibits these effects.

In the antibody experiments, 25 ul aliquots of the cell suspension were added to wells together with 25 ul of antibodies diluted with Dulbecco's phosphate-buffered saline and were then incubated after non-specific binding to the plastic wells had been blocked as described. Measurement of cell attachment and cell spreading was then determined as described above. The results are shown in Table 2 and figure 2 below.

TABLE 2

Spreading (%)	Peptide 1 H1	Peptide 2 VCAM1	Peptide 3 CD45
Control (no antibodies)	19 ±2	16 ±0	18 ±2
Antibody against integrin alpha-4 receptor sub-unit (1:50)	1 ±1	0 ±0	0 ±0
Antibody against integrin beta-1 receptor sub-unit 10 ug/ml	0 ±0	0 ±0	0 ±0
Control IgG 25 ug.ml	19 ±5	10 ±1	19 ±3

The results show that all three peptides were also active in this assay and furthermore that they share the same receptor (which is the integrin heterodimer alpha-4,beta-1).

It will be appreciated that it is not intended to limit the invention to the above example only, many variations, such as might readily occur to one skilled in the art, being possible, without departing from the scope thereof as defined by the appended claims.

CLAIMS

1. A cell adhesion peptide, which may be a polypeptide, including an amino acid sequence comprising X-Asp-Y-(A)n-Phe, where X and Y are chosen from Ala, Leu, Ile and Val, A is any amino acid and n is in the range 3 to 10, in which at least a sub-sequence of the said sequence is adherant for MOLT-4 human lymphoblastic leukaemia, A375-SM human metastatic melanoma or H1080 human fibrosarcoma cells.

2. A peptide according to claim 1, comprising the tripeptide moiety of the sequence "L-D-V" (leucine-aspartic acid-valine).

3. A peptide according to claim 1, comprising the tripeptide moiety of the sequence "L-D-L" (leucine-aspartic acid-leucine).

4. A peptide according to claim 1, comprising the tripeptide moity of the sequence "I-D-A" (isoleucine-aspartic acid-alanine).

5. A peptide according to any one of claims 1 to 4, wherein the number of amino acid units is in the range 15 to 40, and preferably in the range 20 to 30.

6. Pharmaceutical compositions comprising at least one peptide as claimed in any one of claims 1 to 5, with one or more pharmaceutically acceptable exipients and/or diluents, for example normal saline.

7. Method for the modification or control of the adhesive properties of biological cells which comprises treating them with one or more of the new compounds claimed in any one of claims 1 to 5 or a composition as claimed in claim 6.

8. Method according to claim 7, which is used for the treatment or control of inflammatory conditions.

ATTACHMENT OF MOLT-4 LEUKAEMIA CELLS TO "LDV" PEPTIDE-IgG CONJUGATES

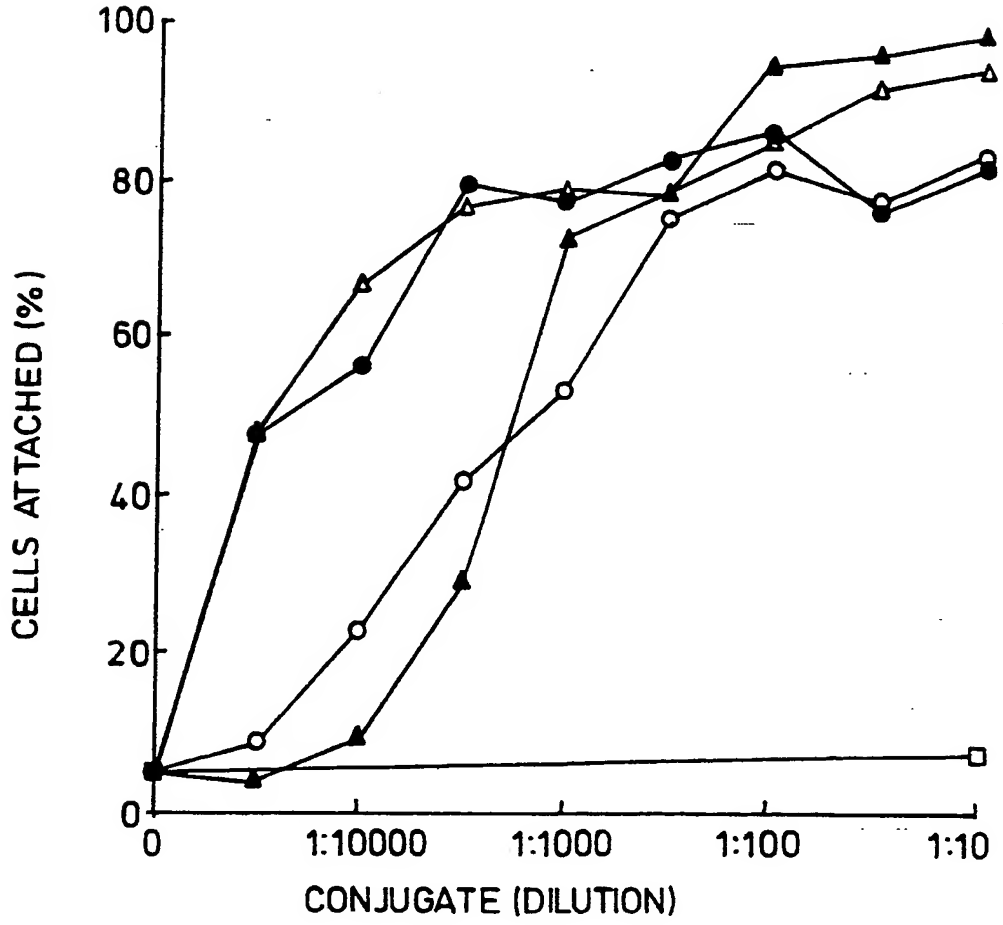

FIG. 1

EFFECTS OF ANTI INTEGRIN ANTIBODIES ON CS12-AND CD45-MEDIATED ADHESION

ANTIBODY	SPECIFICITY	MOLT-4 ATTACHMENT (%)	
		CS12	CD45
NONE		60·6±3·8	75·6±9·8
1:20 8F2	α4	16·8±2·2	30·5±10·5
1:20 CONTROL ASCITES		51·3±5·4	65·5±2·9
25µg/ml MAb 13	β1	4.3±3.0	0±0
25 ug/ml MAb 16	α5	57·9±7·4	55·6±10·9

		A 375-SM SPREADING (%)	
		CS12	CD45
NONE		44±3	18±2
1:50 8F2	α4	0±0	0±0
1:50 CONTROL ASCITES		34±8	24±5
10µg/ml MAb 13	β1	0±0	0±0
25µg/ml CONTROL RaIgG		45±8	19±3

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00226

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 15/00 A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	The Journal of Biological Chemistry, vol. 262, no. 14, 15 May 1987, (Bethesda, Maryland, US), M.J. HUMPHRIES et al.: "Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion", pages 6886-6892, see the whole document	1-8
P,X	The EMBO Journal, vol. 10, no. 13, December 1991, IRL Press, (Oxford, GB), A.P. MOULD et al.: "Identification of a novel recognition sequence for the integrin alpha4beta1 in the COOH-terminal heparin-binding domain of fibronectin, pages 4089-4095, see the whole document	1-8

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
05-06-1992	24. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Nicole De Bie

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND ~~UNSEARCHABLE~~ INCOMPLETELY SEARCHABLE

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 7-8 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effect of the compounds.

2. [ ] Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

Since almost no fixed element exists in the formula of claim 1, and moreover it is not in the possibility of a normal search examination to ascertain the adherency to the cited cell strains of peptides possibly very far from the scope of the invention, the search has been limited to compounds having the formula of claim 1 as furtherly defined by claims 2-5 and to the preparations containing them. Claims searched incompletely : 1-8

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentances of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.